Inhibiting the coregulator CoREST impairs Foxp3\(^+\) Treg function and promotes antitumor immunity

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Foxp3\(^+\) Tregs are key to immune homeostasis, but the contributions of various large, multiprotein complexes that regulate gene expression remain unexplored. We analyzed the role in Tregs of the evolutionarily conserved CoREST complex, consisting of a scaffolding protein, Rcor1 or Rcor2, plus Hdac1 or Hdac2 and Lsd1 enzymes. Rcor1, Rcor2, and Lsd1 were physically associated with Foxp3, and mice with conditional deletion of Rcor1 in Foxp3\(^+\) Tregs had decreased proportions of Tregs in peripheral lymphoid tissues and increased Treg expression of IL-2 and IFN-\(\gamma\) compared with what was found in WT cells. Mice with conditional deletion of the gene encoding Rcor1 in their Tregs had reduced suppression of homoeostatic proliferation, inability to maintain long-term allograft survival despite costimulation blockade, and enhanced antitumor immunity in syngeneic models. Comparable findings were seen in WT mice treated with CoREST complex bivalent inhibitors, which also altered the phenotype of human Tregs and impaired their suppressive function. Our data point to the potential for therapeutic modulation of Treg functions by pharmacologic targeting of enzymatic components of the CoREST complex and contribute to an understanding of the biochemical and molecular mechanisms by which Foxp3 represses large gene sets and maintains the unique properties of this key immune cell.

Introduction

Tregs are essential for maintenance of immune homeostasis and self-tolerance (1, 2). These cells also dampen host antitumor immunity, decreasing the efficacy of tumor immune surveillance (3). The key transcription factor Foxp3 has a critical role in the differentiation and function of Tregs (4, 5), and knockdown or mutations of Foxp3 attenuate the immunosuppressive capacity of Tregs (6, 7). Similarly, depletion of Foxp3\(^+\)CD4\(^+\) Tregs results in severe autoimmunity in otherwise normal animals and can be reversed by reconstituting Tregs (8, 9). Recent successes with checkpoint inhibitor therapies in the treatment of various cancers have rekindled interest in immunotherapy. However, despite a major contribution of Foxp3\(^+\) Tregs has not been studied. Accordingly, we conditionally deleted Rcor1 in Tregs and tested the effects of recently characterized Rcor1 or CoREST inhibitors. We found that gene deletion or pharmacologic inhibition disrupted Foxp3-dependent recruitment of the CoREST complex to the promoters of T-bet, IL-2, and IFN-\(\gamma\), leading to Treg production of IL-2 and IFN-\(\gamma\), impaired Treg function, and enhanced antitumor immunity.

Results

Foxp3, Rcor1, and the CoREST complex. Foxp3 has a central role in maintaining Treg stability and function and forms multiprotein
complexes (≥400–800 kDa) that include various transcription factors and repressor complexes (22), though the functions of these evolutionarily highly conserved repressor complexes are largely unexplored. We analyzed the roles in Tregs of the CoREST scaffolding proteins Rcor1 and Rcor2 and their associated enzymes Hdac1, Hdac2, and Lsd1. In 293T cells transfected with tagged constructs, IP of Foxp3 led to co-IP of Rcor1 (44 kDa), but not Rcor2 (53 kDa, Figure 1A). In reciprocal studies, IP of Rcor1 led to co-IP of Foxp3 as well as Rcor2 and p300 (Figure 1B). We have previously shown that IP of Foxp3 led to co-IP of Rcor1 (44 kDa), but not Rcor2 (53 kDa, Figure 1A). In reciprocal studies, IP of Rcor1 led to co-IP of Foxp3 as well as Rcor2 and p300 (Figure 1B). We have previously shown that IP of Foxp3 leads to co-IP of Rcor1, which is important for Foxp3 acetylation, dimerization, and Treg function (23). The association of Rcor1 with Foxp3 was also demonstrated using Tregs; IP of Rcor1 led to co-IP of Foxp3 (Figure 1C), and IP of Foxp3 led to co-IP of Rcor1 (Figure 1D). Hence, Rcor1 can associate with Foxp3 and with Rcor2 as part of the CoREST complex.

We next conditionally deleted Rcor1 in Foxp3 Tregs by crossing Rcor1fl/fl and Foxp3YFP/Cre mice (Figure 1E). Rcor1fl/flFoxp3YFP/Cre (hereafter Rcor1–/–) mice were born at expected Mendelian ratios and, upon monitoring for up to 1 year, developed normally, without overt development of weight loss, dermatitis, lymphadenopathy, splenomegaly, histologic abnormalities, or other evidence of autoimmunity. To determine whether Treg deletion of Rcor1 affected Foxp3 association with the CoREST complex, we undertook IP of Lsd1 and Western blotting of immunoprecipitates for Foxp3. Unlike in WT Tregs, the association of Foxp3 with the Lsd1 was largely lost in Rcor1–/– Tregs (Figure 1F).

To further characterize the Rcor1–/– mice, secondary lymphoid tissues were harvested, single-cell suspensions prepared, and cell populations and activation markers assessed by flow cytometry. Rcor1–/– mice showed moderately decreased proportions of Foxp3+CD4+ Tregs within lymph nodes and spleen (Figure 2A), and there were only a few significant differences in basal T cell activation markers in lymph nodes, spleen, or thymus, including increased proportions of splenic CD69+CD8+ T cells, decreased proportions of splenic CD4+CD69+ T cells, and increased levels of CD44hiCD62Llo CD8+ T cells in lymph nodes (Figure 2B). The in vitro suppressive functions of Tregs from Rcor1–/– mice were significantly (though modestly) impaired (P < 0.05) compared with those of WT Tregs (Figure 2C, quantified in Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI131375DS1). Hence, Foxp3 can associate with Rcor1 and Lsd1 components of the CoREST complex, and under basal conditions, Rcor1 deletion leads to decreases in peripheral Treg numbers and Treg suppressive function in vitro.

We wondered whether the lack of a phenotype under steady-state conditions in the Rcor1–/– mice might reflect compensation by Rcor2 and briefly explored this by developing mice with conditional deletion of Rcor2 (Rcor2fl/flFoxp3YFP/Cre) or both Rcor1 and Rcor2 (Rcor1fl/flRcor2fl/flFoxp3YFP/Cre) in their Tregs. Conditional deletion of Rcor2 led to a small decrease in peripheral Treg proportions in peripheral lymphoid tissues similar to that seen with Rcor1 deletion (Supplemental Figure 2A). However, in contrast to what occurred with Rcor1, deletion of Rcor2 led to a modest increase (P < 0.05) in Treg suppressive function (Supplemental Figure 2B). Dual deletion of Rcor1 and Rcor2 decreased thymic Treg production, and their peripheral Tregs had somewhat impaired Treg function (P < 0.05) similar to that seen with Rcor1 deletion (Supplemental Figure 2, A and B). Under steady-state conditions, deletions of Rcor1, Rcor2, or both Rcor1 and Rcor2 in Tregs did not lead to marked changes in activation of CD4+ or CD8+ T cells (Supplemental Figure 2C). These data, while not exhaustive, suggest that Rcor1 plays a dominant role in Tregs that is not able to be duplicated by Rcor2.

Figure 1. Association of Foxp3 with the CoREST complex. (A) In HEK-293T cells transfected with tagged constructs encoding Foxp3 (47 kD), Rcor1 (44 kD), and Rcor2 (53 kD), IP of Foxp3 led to co-IP of Rcor1 but not Rcor2 protein. (B) In HEK-293T cells transfected with the same Foxp3, Rcor1, and Rcor2 constructs as shown in A, plus HA-tagged p300, IP of Rcor1 led to co-IP of Foxp3, Rcor2, and p300. (C) Lysates of Tregs isolated from lymph nodes and spleens of WT B6 mice were subjected to IP using anti-Rcor1 Ab or control IgG; Rcor1 and Rcor1-associated Foxp3 were detected by immunoblotting. (D) Tregs isolated from B6 lymph nodes and spleens after expansion in vivo (ril-2/anti-IL-2, 3 days) were subjected to IP using anti-Foxp3 Ab or control IgG; shown is IB detection of Foxp3 and Foxp3-associated Rcor1. (E) Western blots of Rcor1 and Foxp3 expression in Treg and T cells from WT mice or those with conditional deletion of Rcor1 in their Tregs; β-actin was used as a loading control. (F) IP of Lsd1 from WT Tregs led to co-IP of Foxp3, whereas IP of Lsd1 from Rcor1–/– Tregs led to only trace levels of Foxp3 co-IP.
showed enrichment of genes associated with inflammatory and immune responses in Rcor1–/– versus WT Tregs (Figure 3F). Treg expression of cytokines such as IL-2 and IFN-γ is normally highly suppressed, and epigenetic mechanisms are thought to contribute to such regulation. Hence, we used ChIP analysis to assess chromatin remodeling at relevant gene promoters as a result of Rcor1 deletion. We pulled down chromatin with antibodies directed against Lsd1, Hdac1, Hdac2, or acetylated histone 3 (ac-histone 3) and analyzed by qPCR the levels of the promoters of IL-2, IFN-γ, and T-bet that were co-immunoprecipitated. Compared with WT Tregs, Rcor1–/– Tregs had dramatic decreases of Hdac2 and LSD1, no significant difference in HDAC1, and a marked increase of ac–histone 3 at the IL-2 promoter (Figure 4A). Likewise, Rcor1–/– Tregs showed decreased Lsd1/Hdac1/Hdac2 and increased ac–histone 3 binding at the IFN-γ promoter (Figure 4B). IFN-γ is a signature cytokine of CD4+ Th1 cells, and its expression is regulated by T-bet (T-box1). Rcor1–/– Tregs showed decreased Hdac1/Hdac2 and increased ac–histone 3 binding at the T-bet promoter, but there was no significant difference in Lsd1 (Figure 4C). Recent studies have highlighted the crossregulation of IFN-γ and STAT1 with β-catenin, including in dysfunctional Foxp3+ Tregs (24, 25). Although Rcor1–/– Tregs did not affect expression of T-bet and β-catenin proteins under basal conditions, their protein levels were significantly increased in Rcor1–/– Tregs upon activation by CD3/CD28 mAb–coated beads for 24 hours (Figure 4D). These data point to a key role of the CoREST complex in suppressing Treg production of cytokines that are characteristic of activated conventional T cells and only dysfunctional Tregs.

Rcor1 deletion disrupts the Hdac/Lsd1/CoREST complex in Tregs. The CoREST complex, whose primary components are Hdac1 or its paralog Hdac2, Lsd1, and the scaffolding protein CoREST/Rcor1, regulates chromatin remodeling and gene expression (14, 26, 27). Upon studying Rcor1 protein expression in Tregs, we found that Rcor1 was located in cytoplasm under basal conditions in WT Tregs, but after activation with CD3/CD28 mAbs for 24 hours, Rcor1 translocated to the nucleus (Figure 5A), consistent with reports that phosphorylation of Rcor1 in T cells and other cells can lead to nuclear translocation of cytoplasmic Rcor1 (28, 29). Next, we found that the levels of Hdac2 and Lsd1 proteins, but not those of Hdac1, were significantly decreased in Rcor1–/– Tregs (Figure 5B). The best studied modification of core histones is the reversible acetylation of conserved lysine residues within N-terminal tails, as regulated by histone acetyltransferases (Hats) and Hdaccs. A well-established feature
indicate that Rcor1 is important to maintaining CoREST complex-dependent functions in Tregs.

Rcor1 deletion disrupts Treg function in vivo and promotes antitumor immunity. We used 3 animal models to assess the effects of Rcor1 deletion on Treg function in vivo (23, 30). First, we tested the ability of Tregs to inhibit homeostatic proliferation of conventional T cells over 7 days following their adoptive transfer into immunodeficient mice. Cotransfer of WT Tregs significantly inhibited T effector (Teff) cell proliferation, whereas Rcor1 –/– Tregs were less able to suppress Teff cell proliferation (P < 0.05, Figure 6A) and showed upregulation of IFN-γ production (Supplemental Figure 6A). Analogous adoptive transfer with follow-up at 30 days again showed markedly greater expansion of Teff cells in the presence of Rcor1 –/– vs. WT Tregs and suggested that decreased viability of yellow fluorescent protein–positive (YFP +) Rcor1–/– vs. WT Tregs may contribute to this difference, given the reduced number of viable Rcor1 –/– versus WT Tregs (Figure 6B). Rcor1–/– Tregs also showed increased production of IL-2 and IFN-γ compared with WT Tregs (Supplemental Figure 6B).

In a second in vivo test of Rcor1 deletion in Tregs, we undertook cardiac allografts and treated recipients with CD40L (CD154) mAbs plus donor splenocyte transfusion (DST) (5 × 10^6). This well-established costimulation blockade protocol (31) induced long-term allograft survival in WT but not in Rcor1 –/– recipients (Figure 6C), indicating the inability of Foxp3+ Tregs to control host alloresponses in the absence of Rcor1.

Third, we assessed whether Rcor1 deletion in Tregs promoted antitumor immunity, using TC1 and AE.17 lung tumor models. We have previously shown that the growth of these tumors in syngeneic C57BL/6 mice is Treg dependent (23, 32). Compared with WT mice, Rcor1 –/– mice displayed a profound reduction in AE.17 tumor growth (Figure 6D). Flow cytometry analysis showed that Rcor1 –/– mice had increased tumor infiltration by IFN-γ+CD8+ T cells (Figure 6E), and qPCR analysis showed increased CD4, CD8, IFN-γ, and granzyme-B mRNAs and decreased Foxp3 mRNA in tumors harvested from Rcor1 –/– versus WT mice (Figure 6F). Related studies in the TC1 lung tumor model showed that, compared with WT controls, Rcor1 –/– mice had decreased tumor growth (Figure 6G), increased tumor infiltration by IFN-γ+CD8+ T cells (Figure 6H), and increased CD4, CD8, IFN-γ, and granzyme-B mRNAs (Figure 6I). Finally, consistent with Treg dysfunction upon Rcor1 deletion, Rcor1 –/– Tregs produced more cytokines (IL-2, IL-4, IFN-γ) than WT Tregs in tumor-associated lymph nodes and within the tumors themselves (Supplemen-
into Rag1−/− cardiac allograft recipients led to long-term (>100 days) survival in the case of DMSO-treated mice, but resulted in acute allograft rejection in mice treated with JK-2-68 (10 mg/kg/d, 14 days) (*P < 0.01) (Figure 7B) or corin (*P < 0.01, Supplemental Figure 9D). Likewise, the CD40L mAb–based costimulation blockade protocol resulted in long-term (>100 days) allograft survival in DMSO-treated mice, but led to allograft rejection in recipients treated with JK-2-68 (*P < 0.01) (Figure 7C). We also tested the effects of JK-2-68 on histone acetylation and demethylation in Foxp3− Tregs and found that the levels of H3K9Ac and H3K4Me2 were markedly increased (Figure 7D). These results indicate that CoREST complex inhibition impairs the functions of murine Tregs in vitro and in vivo.

**CoREST complex inhibitor impaired human Treg function.** In parallel studies, preincubation of human Tregs with corin impaired Treg suppression of the proliferation of human CD4+ cells (Figure 8A) and CD8+ (Supplemental Figure 12) T cells. By staining cells harvested at the end of these suppression assays for the fixable live/dead marker Zombie and Foxp3 and gating on CD4+CFSE− cells to define “dead Treg” Zombie+ and “live exTreg” Zombie−Foxp3−, and “live Treg” Zombie−Foxp3+ cells, we found that corin exposure had decreased the numbers of live Tregs and increased the numbers of exTregs in these assays (Figure 8B). Statistical analyses of these effects are shown in Figure 8, C and D, respectively. We also noted that corin had decreased Treg expression of Foxp3 and CTLA4 (Figure 8E) and increased expression of CD127 (Figure 8F). Studies of the direct effects of corin on human PBMCs showed that the compound led to decreased proportions of Foxp3+ cells (Figure 8G) and decreased Foxp3 protein expression per Treg (Figure 8H, with statistical analyses in Figure 8, I and J, respectively). Hence, CoREST inhibitor treatment impaired human Treg suppressive function and was associated with decreased expression of Foxp3, CTLA4, and other genes associated with human Treg function.

**CoREST complex inhibitor promotes antitumor immunity.** Although the murine transplant data suggested that CoREST inhibition preferentially affected the functions of Tregs versus Teff cells, since the net effect of systemic compound administration was to induce allograft rejection by host T cells, a far more useful action would be to promote antitumor immunity, consistent with the effects we observed following Rcor1 deletion in Foxp3− Tregs (Figure 6). For these studies, we turned to the use of corin,
a more powerful and pharmacokinetically robust CoREST complex inhibitor than JK-2-68 (33). Importantly, like JK-2-68, corin impaired Treg function in vitro and in vivo (Supplemental Figure 9). Compared with DMSO-treated controls, syngeneic C57BL/6 mice treated with corin had significantly reduced growth of TC1 tumors (Figure 9A). This beneficial effect was accompanied by increased tumor infiltration by effector/memory CD8+ T cells (Figure 9E). No direct effects on tumor growth were seen when tumor-bearing immunodeficient Rag1–/– mice were treated with CoREST complex inhibitor (Supplemental Figure 14). Hence, the inhibitor impaired tumor growth in WT mice by promoting antitumor immunity.

Discussion

Comparative studies of the 3 CoREST genes, encoding Rcor1 and its paralogs Rcor2 and Rcor3, indicate that all 3 CoREST proteins interact equally with Lsd1, but vary in their dependency on Hdac1/2 for their transcriptional repression (35). CoREST complexes containing Rcor1 have the greatest transcriptional repressive capacity (35). In the current studies, pulldown of Foxp3 led to co-IP of Rcor1 but not Rcor2, and pulldown of Lsd1 led to co-IP of Foxp3 only in the presence of Rcor1 (Figure 1). These biochemical data suggest that Rcor1 and Lsd1 are important to Treg biology, consistent with the presence of Foxp3, Rcor1, Hdac1 or Hdac2, and Lsd1, but not Rcor2 or Rcor3, in large multiprotein complexes of 400 to 800 kDa or more in Tregs (22). Under basal conditions, deletion of Rcor1 had only modest effects on Treg development and numbers in secondary lymphoid tissues, and increased tumor infiltration by effector/memory CD8+ T cells (Figure 9E). No direct effects on tumor growth were seen when tumor-bearing immunodeficient Rag1–/– mice were treated with CoREST complex inhibitor (Supplemental Figure 14). Hence, the inhibitor impaired tumor growth in WT mice by promoting antitumor immunity.
ical loss of Treg function when Rcor1−/− mice were challenged by strong T cell activation in vivo. The apparent disconnect between minor effects in vitro and in vivo in Rcor1−/− mice under basal conditions compared with when subject to activating stimuli is not unusual, e.g., the ablation of Blimp1, Icos, IL-10, Ctla4, or Eos in Tregs does not affect their suppressive properties in vitro, but impairs their activation under stimulating conditions and their activities in vivo (36–41), similarly to what occurred in our Rcor1−/− mice. These data and our related findings using bifunctional CoREST inhibitors in WT mice underscore the potential benefit of targeting this complex for therapeutic purposes, such as in cancer immunotherapy.

Figure 6. Rcor1 deletion impairs Treg function in vivo. (A) The ability of Rcor1−/− Tregs (0.5 × 10⁶) to dampen homeostatic proliferation at 7 days after adoptive transfer of Teff cells (1 × 10⁶) into Rag1−/− mice was significantly decreased compared with the effects of corresponding numbers of WT Tregs (P < 0.05). (B) The stability of YFP+ Rcor1−/− Tregs (1 × 10⁶) at 4 weeks after adoptive transfer of Teff cells (0.25 × 10⁶) in Rag1−/− mice was significantly decreased compared with the effects of corresponding WT Tregs, as shown by flow cytometric evaluation of viable cells. *P < 0.05; ***P < 0.001. (C) WT or Rcor1−/− mice (5 mice/group) received BALB/c cardiac allografts plus CD40L mAb/DST; long-term allograft survival was seen in WT but not Rcor1−/− recipients (P < 0.01). (D–I) Treg-specific deletion of Rcor1 enhanced antitumor immunity. Tumor volumes and AUC data of AE17 (D) and TC1 (G) lung tumors were smaller in syngeneic Rcor1−/− vs. WT mice (n = 8–10/group) after inoculation and reached statistical significance (P < 0.05). Analysis of CD4+Foxp3+, CD4+, CD8+, and CD8+IFN-γ+ cells in lymphoid tissues from Rcor1−/− or WT mice, bearing AE17 (E) or TC1 (H) tumors. qPCR analysis of gene expression of CD4, CD8, IFN-γ, granzyme B, and Foxp3 in tumor samples of AE17 (F) or TC1 (I) harvested at the end of each experiment. Data are shown as mean ± SD, 4–6 samples/group. Student’s t test for unpaired data. *P < 0.05; **P < 0.01; ***P < 0.005; ****P < 0.001 vs. WT control.
Deletion of Rcor1 in Tregs increased the expression of multiple transcription factors, cytokines, chemokines, and their receptors, especially upon cell activation (Figure 3). Prominent among these effects were the induction of IL-2 and IFN-γ production by Rcor1−/− Tregs, consistent with decreased recruitment of Lsd1, Hdac1 and/or Hdac2 to the Il2, Ifng, and T-box1 gene promoters and increased histone-3 acetylation at these sites (Figure 4). A key role of the CoREST complex in normally suppressing these events was further supported by the findings of increased H3K4Me2 and H3K9Ac in Rcor1−/− versus WT Tregs, consistent with decreased actions of Lsd1 and Hdac1/2, respectively, as well as by reversal of these features upon overexpression of Rcor1 (Figure 5). Tregs are sensitive to changes in the amount of IL-2 produced by CD4+Foxp3+CD44hi T cells, and this is thought to be a mechanism by which Treg abundance can be rapidly altered as the number of Teff cells fluctuates (42). Likewise, Foxp3 binds and prevents the expression of effector cytokine genes in Tregs (43). The disruption of the epigenetic regulation of IL-2, IFN-γ, and other genes in Tregs as a result of Rcor1 deletion points to additional levels of control and potential for therapeutic intervention beyond regulation of the activation of transcription factors, their DNA binding, and recruitment of transcriptional complexes, as shown schematically in Figure 10.

The in vivo effects of Rcor1 deletion in Tregs were more potent than anticipated by our in vitro data, but are consistent with a reduced transcriptional repressive capacity compared with WT Tregs and decreased suppression of potent Teff responses induced by exposure to allogeneic cells and tumor antigens. We have shown that the ability of AE.17 and TC1 tumor cells to grow in syngeneic C57BL/6 mice is dependent upon the ability of Foxp3+ Tregs to suppress host Teff responses, especially that of CD8+ T cells producing effector molecules such as IFN-γ and granzyme-B (23, 32).

Figure 7. CoREST complex inhibitor affects Treg gene expression and function in vitro and in vivo. (A) qPCR analyses of indicated gene expression in Teff cells and Tregs. qPCR data were normalized to 18S, and data (mean ± SD) are representative of 2 independent experiments involving 5 mice/group. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs. WT control. (B) Immediately after cardiac allografting from BALB/c donors, recipient C57BL/6 Rag1−/− mice (5 mice/group) were adoptively transferred with 1 million B6 Teff cells and 0.5 million B6 Tregs and treated with or without JK-2-68 (10 mg/kg/d, 14 days). P < 0.01 for 2 groups. (C) B6 recipients were transplanted with BALB/c cardiac allografts (5 mice/group) and treated with CD40L mAbs (200 μg)/DST and JK-2-68 (10 mg/kg/d, 14 days). P < 0.01 for 2 groups. (D) Representative bands (left) and statistical analysis (right) of Western blotting for H3K4Me2 and H3K9Ac expression in fresh Tregs or Tregs stimulated for 24 hours by CD3/CD28 mAb-coated beads with or without JK-2-68 (10 μM).
of multiple genes within Foxp3+ Tregs than with effects on Treg trafficking or survival at tumor sites.

Encouraged by the in vivo data following Rcor1 targeting in Tregs, we turned to testing of recently characterized bifunctional CoREST inhibitors (33) in WT mice. These compounds were known to inhibit the proliferation of various tumor lines in vitro as well as having inhibitory effects on the growth of SK-MEL-5 melanoma cell xenografts in immunodeficient mice (33). However, their utility in syngeneic models has not been reported. Since in vivo use of these compounds could have effects beyond just Tregs, we first focused on testing effects of one such CoREST complex inhibitor, JK-2-68, on resting or activated Tregs and conventional T cells. In Foxp3+ Tregs, the inhibitor decreased expression of several signature genes, including genes encoding CTLA4, Foxp3, GITR, and TGF-β, increased IFN-γ and IL-17 expression, and resulted in increased H3K4Me2 and H3K9Ac levels. In conventional T cells, the compound increased IL-2 and IFN-γ production. Hence, the overall brunt of the effects was to dampen Treg genes...
Figure 9. CoREST complex inhibitor enhances antitumor immunity. (A) TC1 tumor volumes and AUC data were smaller in C57BL/6 mice treated with corin (10 mg/kg/d) vs. DMSO (n = 8–10/group). Analysis of the percentages of (B) CD4+ and CD8+ cells, (C) CD8+IFN-γ+ cells, (D) CD4+Foxp3+ cells, and (E) T cell activation markers (CD8+CD69+, CD4+CD69+, CD4+CD44hiCD62Llo, CD8+CD44hiCD62Llo) in lymph nodes, spleens, and tumors from corin- and DMSO-treated groups. Data are shown as mean ± SD, 8–10 samples/group. Student’s t test for unpaired data. *P < 0.05; **P < 0.01 vs. control.
and promote Th1 responses. The histone mark targeted by Lsd1, H3K4Me1/2, is typically associated more with enhancers than promoters. Hence, the observed effects of CoREST inhibition on gene expression could well be mediated by changes at enhancer sites rather than by direct effects at the various gene promoters.

Consistent with its immune-activating effects, JK-2-68 therapy in 2 distinct allograft models in which Treg function is required for long-term transplant survival resulted in allograft rejection. Finally, the more powerful and pharmacokinetically robust compound corin also impaired Treg suppressive function, markedly reduced growth of TC1 tumors in syngeneic mice, and was associated with increased host effector responses, including infiltration by activated CD8+ T cells producing IFN-γ. Although understanding the mechanisms underlying these events is likely more nuanced than what can be found in studies using conditional deletion of Rcor1 in Tregs, the transplant data indicate that the brunt of the effects of JK-2-68 or corin therapy was on the Tregs more than conventional T cells, given that the compound induced acute rejection in Treg-dependent models. Likewise, the cellular and molecular events in Treg-dependent tumor models in Rcor1−/− mice and inhibitor-treated mice have many similarities. In conclusion, while further studies of the roles of Rcor2 and Rcor3 in Tregs and immune responses generally are warranted, the current work shows that Rcor1 and the CoREST complex have important roles in Foxp3+ Tregs, such that therapeutic manipulation using CoREST complex inhibitors may be of benefit in cancer immunotherapy.

**Methods**

**Mice.** We used WT BALB/c and WT C57BL/6, Rag1−/− C57BL/6, CD90.1/B6, Rcor1−/− (catalog 025877), and Rcor2−/− (catalog 030004) mice from The Jackson Laboratory, plus previously described Foxp3YFP+ YFP− mice (38). All mice were backcrossed on the C57BL/6 background at least 8 times and used at 6 to 8 weeks of age unless specified.

**Plasmids and CoREST complex inhibitors.** We purchased plasmids from Addgene and transiently expressed Flag-myc-tagged-Rcor1, Flag-myc-tagged-Rcor2, Flag-tagged-Foxp3, and HA-tagged-p300 in 293T cells (23). Preparation of CoREST complex inhibitors JK-2-68 and JKD-1-51 (corin) was described previously (33).

**Co-IP and Western blotting.** HEK-293T cells transfected with plasmids, as well as WT and Rcor1−/− Tregs, were lysed with RIPA buffer (MilliporeSigma, catalog SLB1395V). Pulldown Abs were incubated with precleared samples for 2 hours at 4°C, then overnight with protein G-agarose (Invitrogen, catalog 15920-010). Cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted with the indicated Abs. We purchased Abs against Rcor1 (Millipore, catalog MABN486), Foxp3 (Invitrogen, catalog 700914; eBioscience, catalog 14-4774-82), and histone H3 (Abcam, catalog ab1791) as well as Flag (catalog 14793), Myc (catalog 2272), HA (catalog 2367), Rcor2 (catalog ab37113), Lsd1 (catalog 2139), β-actin (catalog 3700), H3ac (catalog 34589), H3bd (catalog 57156), Pcaf (catalog 3378), T-bet (catalog 13232), β-catenin (catalog 8480), H3K9ac (catalog 9649), H3K4m2 (catalog 9725), and ac-histone 3 (catalog 97549) from Cell Signaling Technology. Secondary HRP-conjugated Abs to mouse (catalog 7076), rat (catalog 7077) and rabbit (catalog 7074) IgG were purchased from Cell Signaling Technology. Unconjugated CD3 (clone 145-2C11, catalog 553057) and CD28 (clone 37.51, catalog 553294) mAbs used for cell activation were purchased from BD. Full, uncut gels were acquired on a Cytoflex (Beckman Coulter) flow cytometer.

**Murine Treg suppression assays.** For in vitro studies, 5 × 10^4 cell-sorted CD4+CD25− T cells and CD4+CD25+ Tregs from Foxp3YFP− and Rcor1−/− mice isolated using the CD4+CD25+ Treg Isolation Kit (Miltenyi Biotec, catalog 130-091-041) were added to 96-well plates. Equal numbers of CFSE-labeled CD4+CD25+ T cells and γ-irradiated APCs, isolated using a CD90.2 kit (Miltenyi Biotec, catalog 130-049-101) plus CD3ε mAb (1 μg/mL) were cultured for 72 hours. After 72 hours, proliferation of Teff cells was determined by flow and analysis of cell trace violet dilution. For in vivo Treg suppression assays, 1 × 10^6 CD4+CD25− Thy1.1+ and 0.5 × 10^6 Tregs were injected i.v. into Rag1−/− mice. At 1 week, lymph node and spleen cells were stained with Thy1.1-PE and CD4–Pacific Blue, and the numbers of Thy1.1+ T cell determined (Cytoflex).
press divisions of CD4+ and CD8+ T cell responders was analyzed separately. Five independent experiments were performed using 5 different healthy donors of normal Tregs and 3 different healthy donor responders, and most assays used pretreatment of Tregs with 1 μM of corin, such that data from 12 suppression assays were collected.

In addition, we assessed the effects of corin on human PBMCs. PBMCs from 5 healthy donors were incubated overnight with corin (1 μM) and CD3ε/CD28 mAb–coated beads (1.3 beads/cell) and analyzed the next day by flow cytometry. In total, 18 markers were evaluated in CD45-Ghost CD4+Foxp3+ Tregs (IFN-γ, IL-2, IL-4, IL-17, CD45RA, CD45RO, CD62L, CD69, PTEN, CD39, FAS, CD120b, GITR, TIGIT, CD127, CTLA4, HLA-DR, CD25). For cytokine production, PBMCs were stimulated the next day for 4 hours with PMA/ionomycin in the presence of Brefeldin. Human healthy donor Tregs did not produce substantial amounts of cytokines, so we noted only the markers whose expression was altered by the presence of corin.

Cardiac transplantation. Heterotopic cardiac allografts were performed using BALB/c donors and WT or R1Δ−/− recipients (C57BL/6 background), as described (30). In adoptive transfer studies of Treg-dependent allograft survival, after their isolation using magnetic beads, Tregs (0.5 × 10^6) from WT or R1Δ−/− mice and Teff cells (1 × 10^6) from WT mice were injected i.v. into R1Δ−/− mice bearing BALB/c cardiac allografts. In studies of costimulation blockade-dependent allograft tolerance, WT or R1Δ−/− allograft recipients were treated at the time of engraftment with CD154 mAbs plus 5 × 10^6 donor splenocytes (31). Graft survival was monitored as a function of the ability of Tregs to suppress T eff cell–dependent alloreactivity and cardiac allograft rejection. In related pharmacologic studies, R1Δ−/− allograft recipients receiving adoptive transfer or WT recipients treated with CD154 mAbs were treated with CoREST inhibitors (10 mg/kg/d, 14 days) from engraftment.

ChIP assays. We purchased EZ-Magna ChIP A Chromatin Immunoprecipitation Kits (Millipore). T eff cells or Tregs were fixed with 1% formaldehyde and fragmented by sonication. Chromatin was immunoprecipitated with Abs against acetyl-histone-H3, Hdac1, Hdac2 and Lsd1, and the resultant DNA was purified and analyzed by real-time PCR (Step-One, Applied Biosystems). Il2 and Ifng primer sets were reported previously (45); primers for T-box1 Il2 and noprecipitated with Abs against acetyl-histone-H3, Hdac1, Hdac2 and Lsd1-WWH oversaw experimental design and writing of the manuscript.

Statistics. Data were analyzed using GraphPad Prism 8.0. Data are presented as mean ± SD unless specified otherwise. Measurements between 2 groups were done with 2-tailed Student’s t test when data were normally distributed or Mann-Whitney U unpaired test when populations were not normally distributed. Groups of 3 or more were analyzed by 1-way ANOVA with corresponding Tukey’s multiple-comparisons test when normally distributed, or when not, using Kruskal-Wallis with Dunn’s multiple-comparisons test. Graft survival was evaluated with Kaplan-Meier followed by log-rank test; P < 0.05 was considered significant. For human Treg suppression assays, AUCs were calculated as described (44), followed by calculation of the ratios of AUCs to control Treg AUCs. For data presented as ratios, 1 sample t test (theoretical mean = 1) was applied for normally distributed data; otherwise, we applied Wilcoxon’s signed rank test (theoretical median = 1). When needed, all P values were corrected with Bonferroni’s test for multiple comparisons. Foxp3 median of fluorescence data were calculated using unpaired Student’s t test.

Study approval. Animal studies were approved by the Institutional Animal Care and Use Committee of the Children’s Hospital of Philadelphia (protocols 17-001047 and 19-000561).

Author contributions. YX designed and performed experiments and drafted the manuscript. LW performed cardiac transplants and performed experiments. RH provided technical assistance. EDG performed experiments. UHB provided assistance with RNA-Seq studies. TA performed studies. MT performed studies. JHK provided assistance with CoREST complex inhibitors. PAC provided assistance with CoREST complex inhibitors and assistance with the manuscript. WWH oversaw experimental design and writing of the manuscript.
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